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Award Number: W81XWH-12-2-0076

TITLE: Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections.

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REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Oct/01/2013	Annual	30 Sep 2012 to 29 Sep 2013

4. TITLE AND SUBTITLE

Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-2-0076

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Public Health Research Institute
New Jersey Medical School
Rutgers, The State University of New Jersey
Newark, New Jersey 07103

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the first year of this research contract we demonstrated the antimicrobial properties of Carbohydrate-Derived Fulvic Acid (CHD-FA) against a broad collection of multi-drug resistant bacterial and fungal pathogens commonly associated with wound infections. We completed the objectives in specific aim 1 of the award statement of work by determining the in vitro susceptibility of CHD-FA against a collection of multi-drug resistant Acinetobacter baumanii, Pseudomonas aeruginosa, carbapenamase-resistant Klebsiella pneumoniae, Enterococcus faecium, Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus (MRSA), Escherichia coli, Enterobacter spp., other clinically important species. as well as azole resistant fungal pathogens Candida spp., Aspergillus fumigatus and polyene-resistant non-fumigatus Aspergillus species, Fusarium species and Zygomycetes. We have also established a cutaneous wound infection model in rats with MRSA and Pseudomonas aeruginosa to assess (CHD-FA) as a potent topical agent to prevent drug resistant wound infections and promote healing as part of specific aim 2 of the research contract. Daily topical applications of CHD-FA were placed on the cutaneous wounds in rats at 4 and 24 H post infection for up to 10 days. Wound size and wound bacterial burdens were measured to assess the treatment efficacy of CHD-FA. CHD-FA treated animals had improved wound healing and reduced bacterial burden.

15. SUBJECT TERMS
carbohydrate-derived fulvic acid (CHD-FA), drug resistant wound infections,

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	22	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

The objective of this study is to demonstrate the potent antimicrobial properties of carbohydrate-derived fulvic acid (CHD-FA) against a broad collection of drug-sensitive and multi-drug-resistant (MDR) pathogens commonly associated with wound infections, and assess the relative efficacy of CHD-FA against induced wound infections in *in vivo* animal models. Overall, this work is intended to establish CHD-FA as a safe and effective agent that can be deployed to prevent the onset of drug-resistant bacterial and fungal infections in military and civilian personnel suffering traumatic wound infections. Given its novel mechanism of action and preliminary activity against MDR bacteria and antifungal-resistant fungi, the early use of CHD-FA is advantageous because it represents a novel target that will not select for resistant organisms, and prevents the use of more specific but more limited spectrum antibiotics. The overall goal of this preclinical program is to establish a firm justification for progressing to human trials to determine the efficacy of topical CHD-FA in preventing wound infections in injured military personnel.

Body

Description of Overall Progress

1. Establish minimum inhibitory concentrations (MIC50 and MIC90) for CDH-FA against large collections of clinical isolates representing wound-associated drug resistant bacteria and fungi.

During the last 3 quarters, we completed specific aim 1 of the statement of work and established the minimum inhibitory concentrations (MIC_{50} and MIC_{90}) for CDH-FA against a collection of over 600 multidrug resistant Gram negative, Gram positive bacterial and fungal clinical isolates. These isolates were obtained through our collaboration with PHRI Principal Investigator Dr. Barry Kreiswirth as part of large molecular characterization (molecular typing, resistance mechanism, sequencing) programs for drug resistance with New York and New Jersey Hospitals. All fungal isolates tested were originally obtained and catalogued from the National Aspergillosis Center in Manchester, UK, and the Perlin Pfizer Reference Center for molecular characterization of echinocandin resistance in yeasts and molds and from the University of Texas Health Science Center at San Antonio Texas. These isolates were derived largely from bloodstream, soft-tissue, burn, wound, pustules, and respiratory fluids specimens. All clinical isolates were blinded for any patient protected health information. All bacterial and fungal isolates were sub-cultured on a semi-solid rich medium to prepare fresh samples for the susceptibility testing. Frozen stocks from the sub-cultured isolates were prepared and catalogued.

A highly standardized broth-based in vitro susceptibility assay following the Clinical and Laboratory Standards Institute (CLSI) protocol M07-A9 “Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Eighth Edition” (1) was used to determine the CHD-FA MIC values for all the bacterial strains, while the CLSI protocol M38-A2 “Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard” (2) was used to determine the CHD-FA MIC values for all Aspergillus strains, and the CLSI protocol M27-A3 “Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Third Edition” (3) was used to determine the CHD-FA MIC for all Candida strains. Quality control (QC) antibiotic susceptible Gram-negative and Gram-positive bacterial strains from the American Type and Culture Collection and control antibiotics ciprofloxacin and doxycycline were used to ensure testing parameters were conforming to the CLSI methodology. The MIC results with the QC control strains and control antibiotics demonstrated the assay were within the established CLSI range. The methods and QC results have been previously described in our first Quarterly Technical Progress Report (Q1.01.2013) (Appendix).

Detailed procedures and a list of isolates can be found in our Quarterly Technical Progress Reports Q1.01.2013, Q2.04.2013 and Q3.07.2013 (Appendix). **Table 1** summarizes the MIC50 and MIC90 values of the bacterial and fungal isolates at 24, 48 or 72 hours. The concentration of CHD-FA to inhibit the growth of the drug resistant bacteria and fungi ranged from 0.06 to 0.5%. Our results are comparable to the preliminary data in the grant award and previously published reports on CHD-FA MIC values (4).

Conclusion: We have demonstrated the potent anti-microbial activity of CHD-FA against a broad range of drug resistant bacteria and fungi. Most bacteria show complete growth inhibition at 0.06 – 0.125% fulvic acid, while efficacy with fungi is 0.125-0.5%. We will assess potential bactericidal v. bacteriostatic behavior going forward.

Table 1. Average $MIC_{50/90}$ values at 24 and 48 hours for all Gram-negative, Gram-positive and fungal isolates

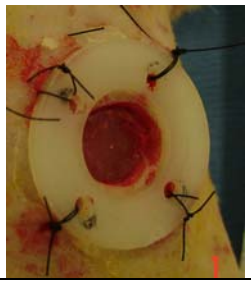
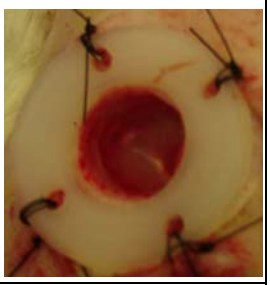
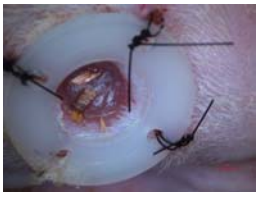
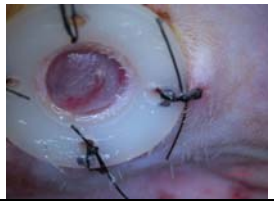
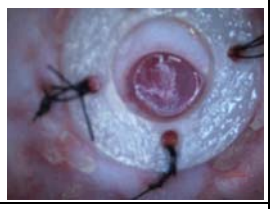
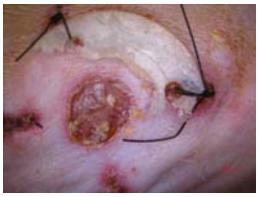
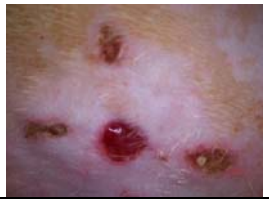
Organism	# of isolates	MIC 24 Hr		MIC 48 Hr	
		MIC_{50} 24Hr (%)	MIC_{90} 24Hr (%)	MIC_{50} 48 Hr (%)	MIC_{90} 48 Hr (%)
Enterobacter cloacae	50	0.125	0.125	0.125	0.125
Enterobacter aerogenes	24	0.125	0.125	0.125	0.125
Escherichia coli	50	0.125	0.125	0.125	0.125
Klebsiella pneumoniae	50	0.125	0.125	0.125	0.125
Pseudomonas aeruginosa	50	0.06	0.125	0.125	0.125
Enterococcus faecium	50	0.06	0.06	0.125	0.125
Methicillin-resistant *Staphylococcus aureus*	50	0.125	0.125	0.125	0.125
Methicillin-susceptible *Staphylococcus aureus*	50	0.125	0.125	0.125	0.125
Streptococcus pyogenes	50	0.06	0.06	0.06	0.06
Aspergillus fumigatus	12	0.5	0.5	0.5	0.5
Aspergillus flavus	12	0.5	0.5	0.5	0.5
Aspergillus terreus	5	0.125	0.125	0.5	0.5
Aspergillus niger	10	0.5	0.5	0.5	0.5
Candida albicans	24	0.5	0.5	0.5	0.5
Fusarium solani	*5*	*0.125*	*0.125*	*0.125*	*0.125*
Absidia corymbifera	*5*	*0.5*	*0.5*	*0.5*	*0.5*
Rhizopus oryzae	*3*	*0.5*	*0.5*	*0.5*	*0.5*
		MIC_{50} 48Hr (%)	**MIC_{90} 48Hr (%)**	**MIC_{50} 72Hr (%)**	**MIC_{90} 72Hr (%)**
Penicillium marneffei	*2*	*0.25*	*0.25*	*0.25*	*0.25*
Penicillium chrysogenum	*2*	*0.25*	*0.25*	*0.25*	*0.25*

2. Establishing the cutaneous wound model in rats (specific aim 2).

The purpose of this pilot trial was to observe natural wound healing process and to establish the kinetics of such process in rats without infection by digital measurement of wound size over time. Six male Sprague Dawley rats (~200g) were anesthetized by intraperitoneal injection of 100mg/kg ketamine +10mg/kg xylazine. The dorsal side of the rats were shaved with electrical clippers and chemically depilated. The exposed skin was wiped with betadine. Using a 0.8cm diameter disposable biopsy punch, two symmetrical wounds were created on the dorsum of each rat. Sterile polyurethane rings were placed over the fresh wounds. The rings were attached with adhesive and also with four nylon microfilament sutures. Wounds were covered with a Tegaderm visible adhesive dressing, and rats were rehydrated with saline administered via subcutaneous injection. The analgesic buprenorphine (0.05mg/kg) was administered twice daily for two days to minimize pain during surgical recovery. Wound healing was observed for 10 days, during which Tegaderm dressings were changed and pictures were taken of the wounds daily.

Figure 1 below shows representative images of the wounds over the course of day 0 (day of surgery) through day 10:

Fig.1. Cutaneous wounds in rats (no infection)

Day	Rat #1	Rat #3	Rat #4
0			
2			
4			
6			

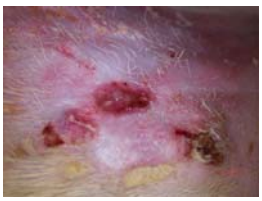
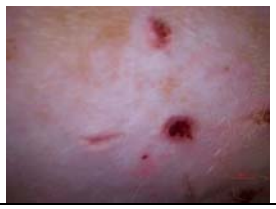
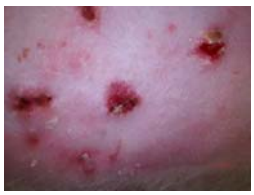
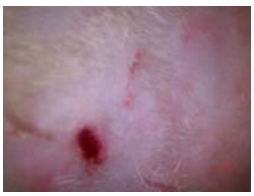
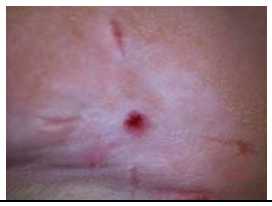
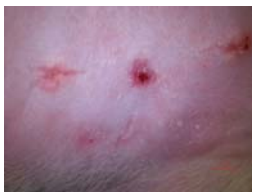
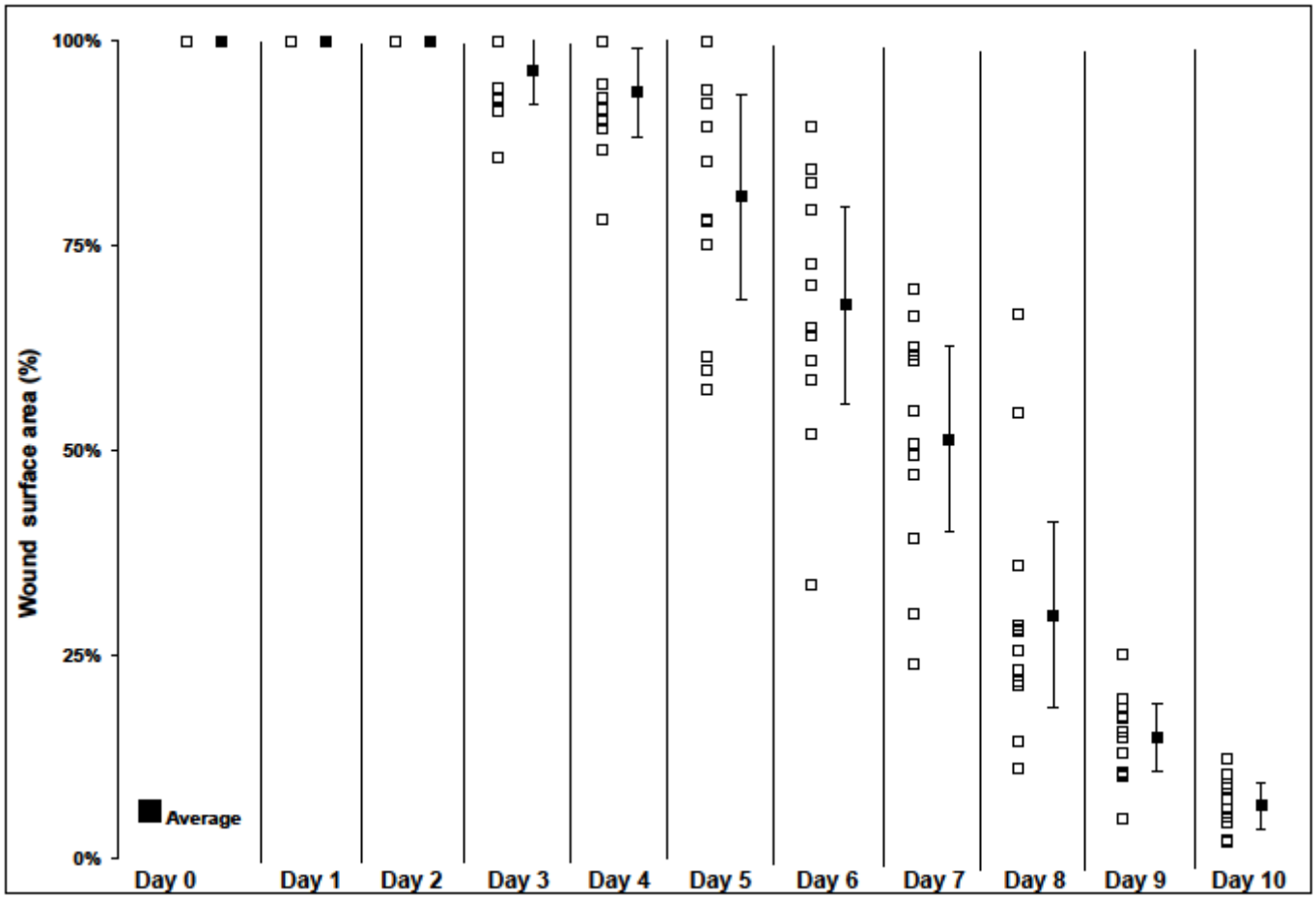
8			
10			

Figure 2 is a scatter plot of wound surface area of the 12 wounds created, over the course of 10 days. Surface area of each wound is measured as a percentage of the surface area of that wound on day 0. By day 10, wound surface areas of all the rats were less than 10%.

Fig.2. Cutaneous wound surface area (Closing) measurements.



Anticipated problems and corrective actions.

Removal of the wound splint and reopening of the wounds by the rats are concern as these activities can significantly influence wound healing. We modified the bandaging and dressing the wounds in rats to allow them enough mobility to eat, drink and groom but limits their access to the wound sites and prevent chewing of the wound splints.

3. Establishing the wound infection in rats with Methicillin Resistant *Staphylococcus aureus* (MRSA) strain Xen31 (specific aim 2).

Nine rats were randomized into three infection groups (3 rats per group). Bioluminescent Methicillin Resistant *S. aureus* (MRSA) strain Xen31 was inoculated in Brain Heart Infusion (BHI) media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to $1x10^{10}$, $2x10^{8}$, and $1x10^{8}$ colony forming units (CFU) per ml for the infection. Two wounds were created on each rat, for a total of eighteen wounds. The process of anesthetizing the rats and creating the wounds was the same as described above in the section on establishing the wound model and in quarterly technical report Q3.07.2013 . After wound creation, rats from each challenge group were infected with 0.05 ml of *the* MRSA cell suspension with corresponding doses. The final infection dose for the rats were 5 $x10^{8}$, $1x10^{7}$, and $5x10^{6}$ CFU, respectively.

The wounds infected with MRSA became purulent starting at day 1. The amount of purulent discharge was dependent on amount of MRSA infection. Scab formation was by day 1 and all wounds had scabs by day 2. Purulent discharge of the wounds was observed at various levels throughout the 10 day experiment **(Fig.3)**. Wound closure in the rats was visible noticeable starting at day 5 at the two lower infection doses ($1x10^{7}$ and $5x10^{6}$ CFU) and by day 6 at the highest dose (5 $x10^{8}$). Interestingly, there were no observable signs of sepsis such as lethargy or recumbency as a result of infection in any of the rats. Daily digital wound measurement were taken with a Nikon 4x Stereomicroscope with FS-1 digital camera. Wound closure as percentage of wound surface area **(see Fig.4.)** was discernible between the highest infection dose and the lower doses from days 6 through 8, however by the experimental endpoint at day 10 the untreated infected wounds were all similar in surface area.

Conclusion

Based on the virulence study, a ~10^8 CFU infection dose will be used for all downstream MRSA cutaneous wound infections.

Fig. 3. Cutaneous wounds in rats with MRSA (Xen31) infection

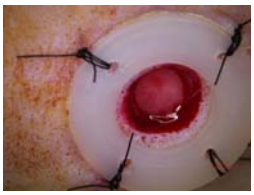
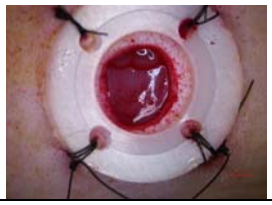
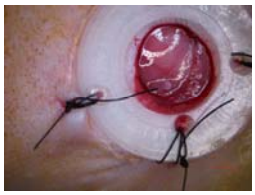
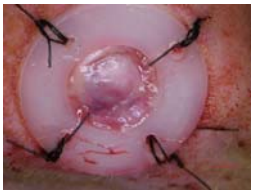
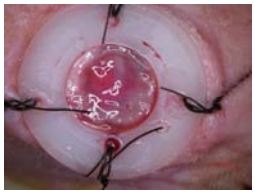
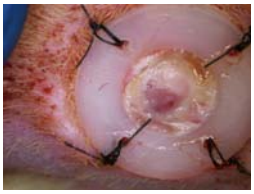
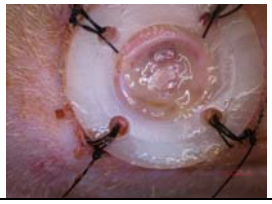
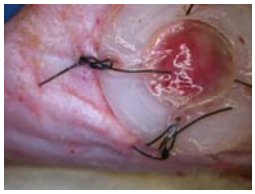
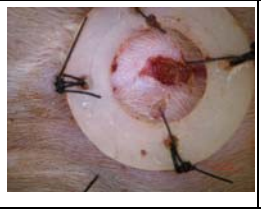
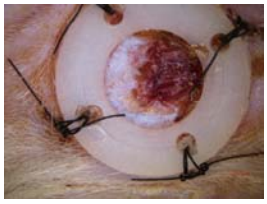
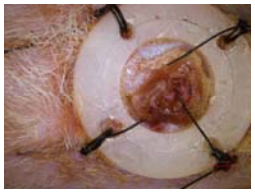
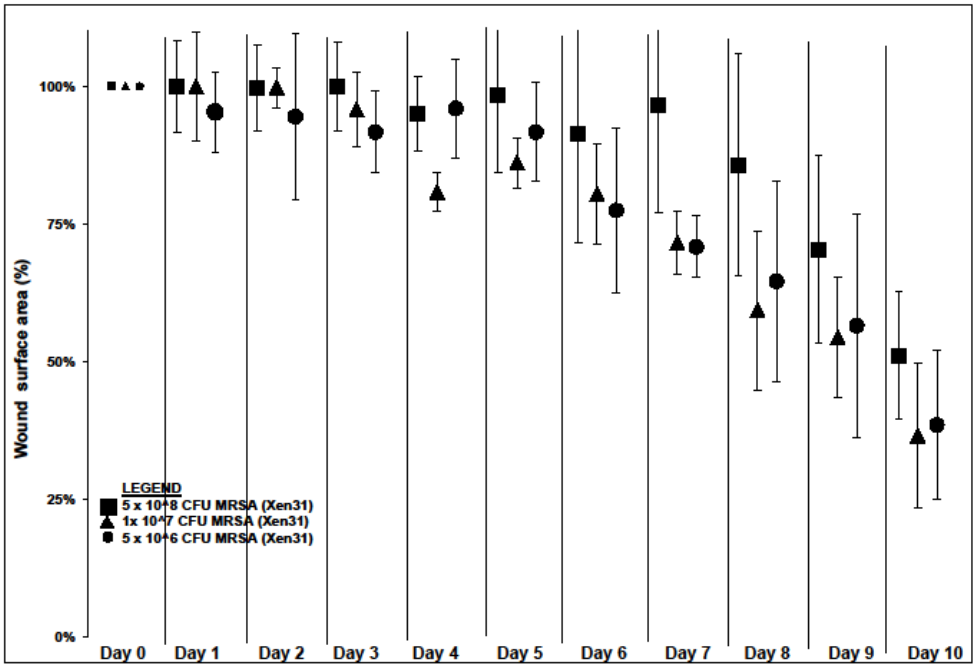
Day	Rat #2 ($5x10^8$ cells)	Rat #5 ($1x10^7$ cells)	Rat #8 ($5X10^6$ cells)
0			
1			
2			
10			

Figure 4. Wound surface are plot of wound infection model with MRSA



4. Establishing the wound infection in rats with *Pseudomonas aeruginosa* strain Xen05 (specific aim 2).

As with the MRSA virulence assessment, 9 rats were randomized into three infection groups (3 rats per group). Bioluminescent *P. aeruginosa* was inoculated in Brain Heart Infusion (BHI) media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to $1x10^{10}$, $2x10^{8}$, and $1x10^{8}$ colony forming units (CFU) per ml for the infection. Two wounds were created on each rat, for a total of eighteen wounds. After wound creation, rats from each challenge group were infected with 0.05 ml of the cell suspension with corresponding doses. The final infection doses for the rats were 1 $x10^{9}$, $1x10^{8}$, and $1x10^{7}$ CFU, respectively.

All the wounds infected with *P. aeruginosa* became purulent starting at day 1. Hemolysis was also observed in the wounds. Purulent discharge of the wounds was observed at various levels throughout the 10 day experiment **(Fig.5)**. However, by day 2 post infection, all the rats from the highest infection dose became moribund and were euthanized. Post mortem analyses of the euthanized rats from the highest infection dose identified *P. aeruginosa* in the blood and wide dissemination to organs such as spleen, kidneys, liver and heart. *P. aeruginosa* was also recovered from both nasal and ocular discharges. Two out of the 3 rats infected with 10^{8} CFU of *P. aeruginosa* became moribund by day 3 post infection and were summarily euthanized. Post mortem analysis had similar results to the euthanized rats on day that were given the highest infection dose.

Rats given $1x10^{7}$ CFU of *P. aeruginosa* did not display any observable signs of septic infection as well as the one rat given 10^{8} CFU. Wound closure was visually noticeable starting at day 5. Wound closure analysis of the digital images of the Rats infected with 10^{7} CFU **(see Fig.6.)** at the experimental endpoint on day 10 had > 25% closure of the untreated infected wounds.

Conclusion

Based on the virulence study, a $1x10^{7}$ CFU infection dose will be used for all downstream *P. aeruginosa* cutaneous wound infections.

Figure 5. Cutaneous wound images of rats infected with varying infection doses of *Pseudomonas aeruginosa (Xen05)*.

Day	Rat #1	Rat #4	Rat #8	Rat#10

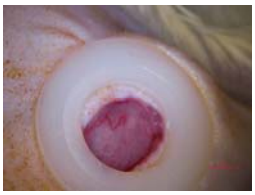
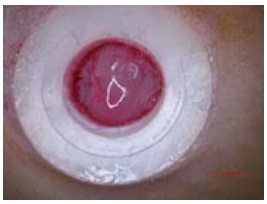
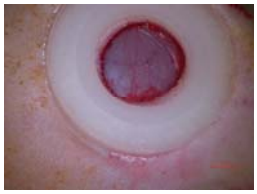
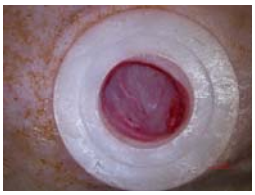
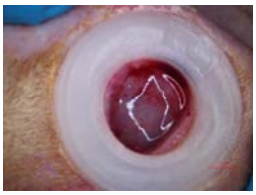
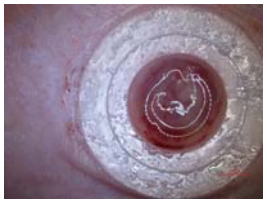
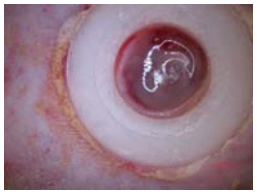
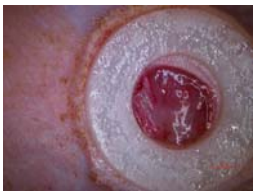
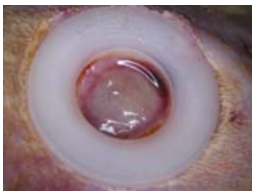
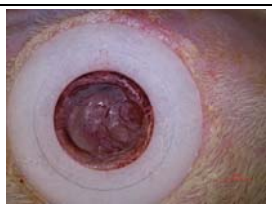
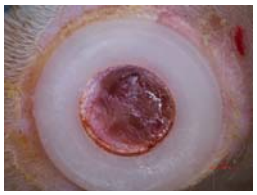
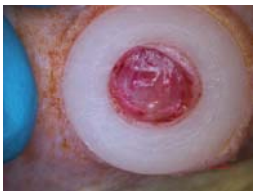
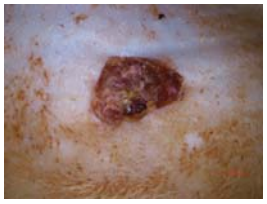
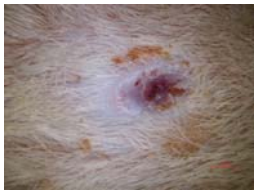
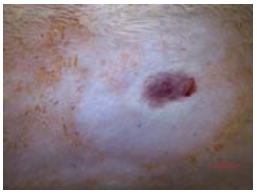
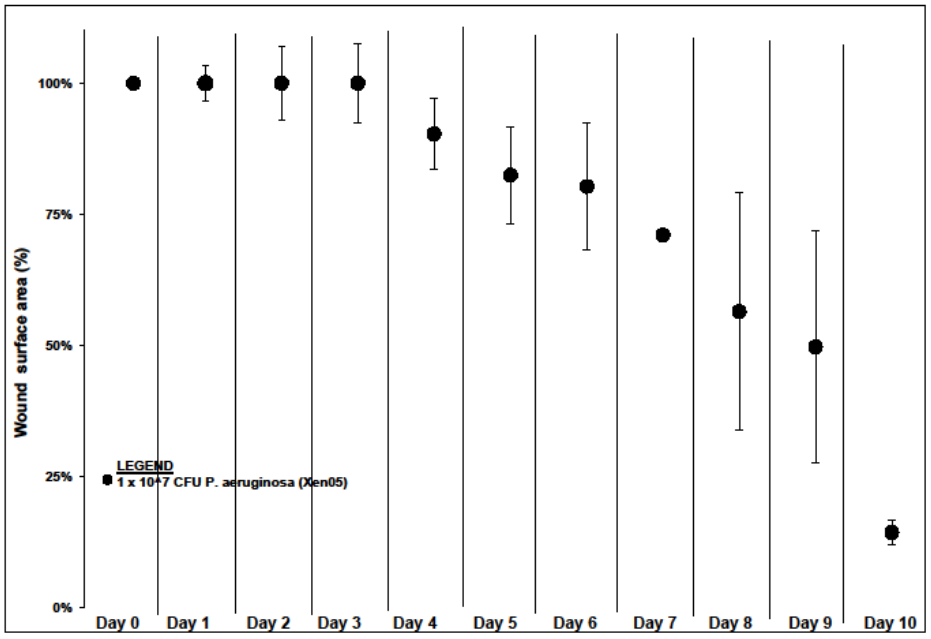
	($1X10^9$ CFU)	($1x10^8$ CFU)	($1X10^7$ CFU)	Uninfected
0				
1				
2				
10	**Euthanized on day 2. see above image**			

Figure 6. Wound surface area plot of cutaneous wound infection model with 1.0 x 10^7 CFU *P. aeruginosa*



5. Evaluation of CHD-FA to treat cutaneous wounds infected with MRSA (specific aim 2).

To assess the treatment efficacy of CHD-FA against cutaneous wounds infected with MRSA, two cohorts of 11 rats were randomized into three treatment groups (3 rats per group) and two untreated

(sham) controls. Bioluminescent MRSA Xen31 was prepared at previously described. Wound were created and infected with 0.05 ml of MRSA to a final infection dose of $1x10^8$ CFU. Rats were given daily treatments of CHD-FA at 4.6, 0.46 or 0.046% in 0.025 ml volumes starting at 4 h or 24h post infection. The 4h and 24h treatment starting time was used to determine if significant improvement in wound healing and infection reduction would be observed. The MRSA infection was a more appropriate model to evaluate this as the virulence study demonstrated the rats could better tolerate MRSA wound infections.

Improved wound closure was visually observed in the highest and middle CHD-FA doses (4.6 and 0.46%) relative to the untreated control in both the 4h and 24h post infection treatment start time studies **(Figs 7 and 8)**. The greatest improvement in wound healing can be seen from day 5 to day 7. As expected, both the CHD-FA treated and untreated wounds were significantly healed by the experimental endpoint at day 10. Purulent discharge was observed in some of the CHD-FA treated and untreated rats regardless of the treatment start time (24h or 4h, post infection), however, the discharge was less pronounced in the CHD-FA treated groups. Digital measurements of wound surface areas **(Table 2)** showed significant improvement in wound healing in the 4h treatment start time than the 24h start in all 3 doses evaluated. The most prominent differences were on day 7 where the average wound surface areas for the 24h treatment start were 56.8%, 59.1% and 64.4% in contrast to the 4 h treatment start of 43.9%, 45.3% and 43% for CHD-FA doses 4.6%, 0.46% and 0.046%, respectively. The wound surface area percentages for the untreated controls on day 7 was 70.4% and 64.5%, for the 24h and 4h treatment start time, strongly suggesting the initiation of CHD-FA therapy is an important factor in improved wound healing. The bacterial burden assessment of the wounds at the experimental endpoint at 4h CHD-FA treatment time had 1.5, 2.1 and 0.3 log reduction in burden for the CHD-FA treatment doses 4.6%, 0.46% and 0.046% relative to the untreated controls **(Table 3)**. Interestingly, the mid-dose CHD-FA (0.46%) had the most pronounced reduction in bacterial burden at 2.1 logs, however based on the changes in the wound surface area sizes on days 5 to 7, we need to assess bacterial burdens in that time frame. Unfortunately, the bacterial burden was too low to count in the 24h treatment groups including the untreated controls at the experimental endpoint.

Conclusion

The significant reduction of the size and bacterial burden in the wound sites demonstrate the potency of CHD-FA to treat wound infections. We will be repeating these studies with a focus on the earlier time points to assess bacterial burden. Furthermore, we will also perform both histopathologic analyses and host gene expression profiling to better assess the cellular and molecular mechanisms during wound healing with CHD-FA.

Figure 7. Wound images of rats infected with MRSA (Xen31) and treated with CHD-FA at 24h post infection.

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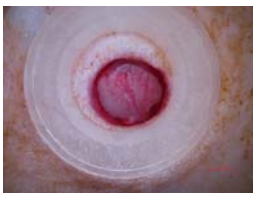
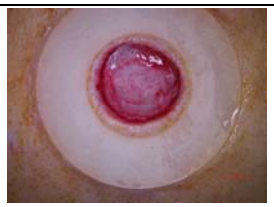
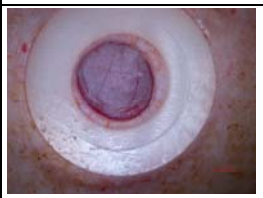
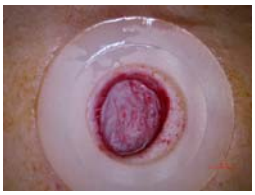
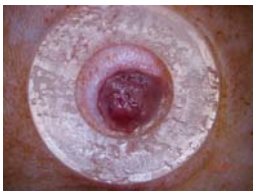
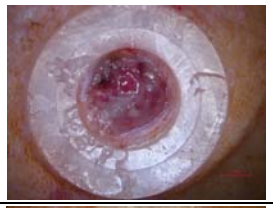
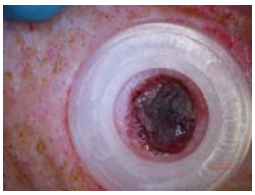
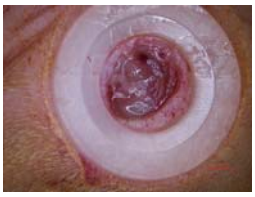
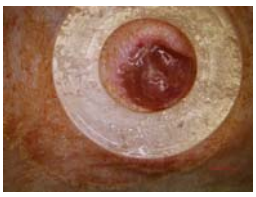
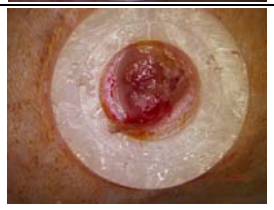
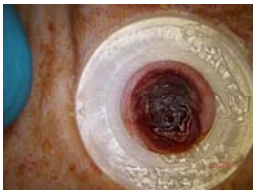
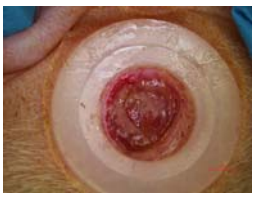
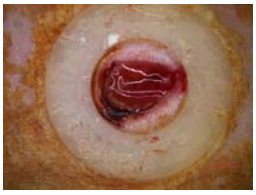
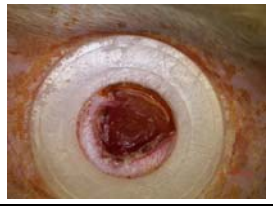
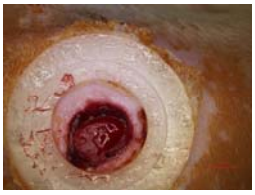
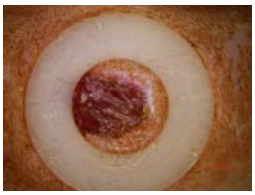
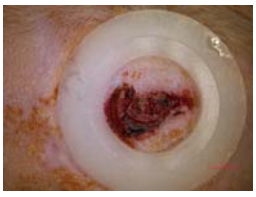
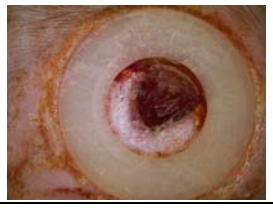
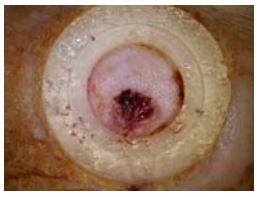
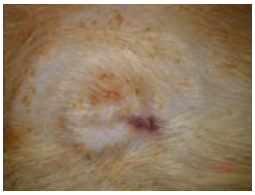
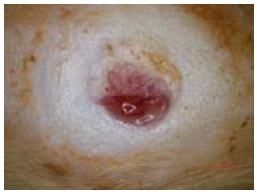
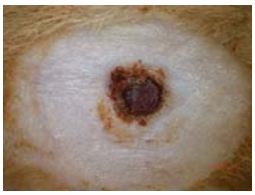
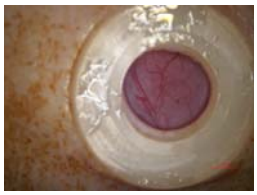
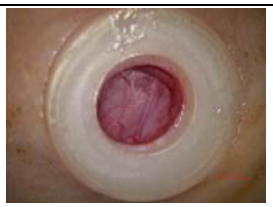
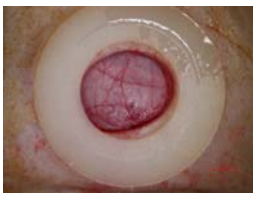
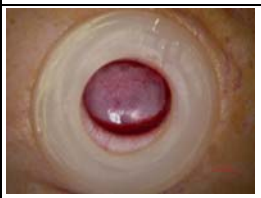
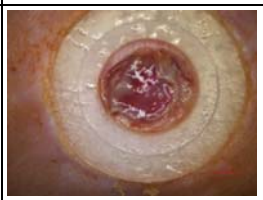
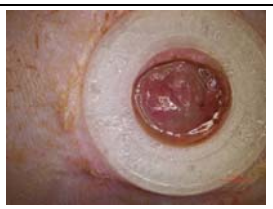
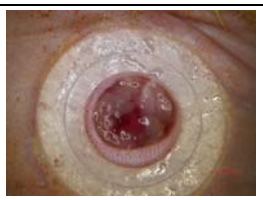
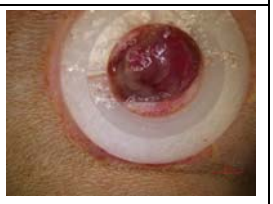
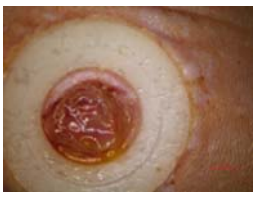
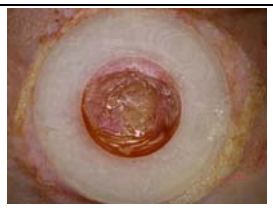
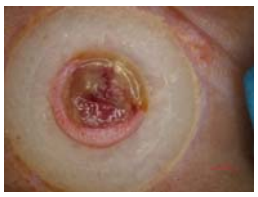
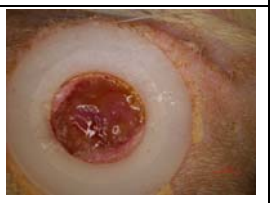
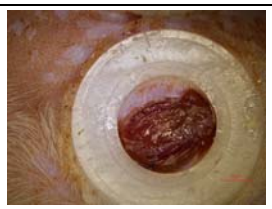
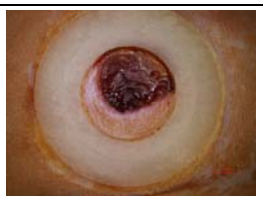
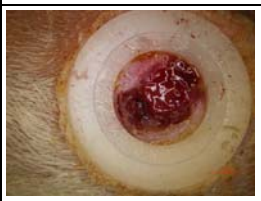
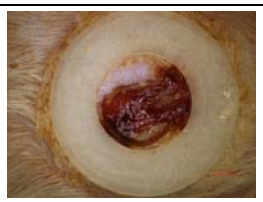
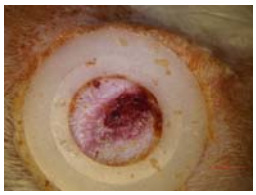
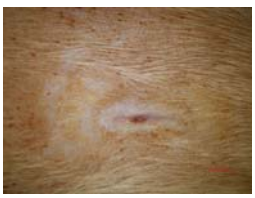
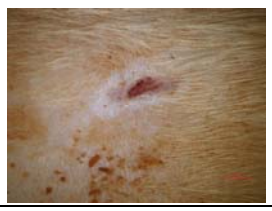
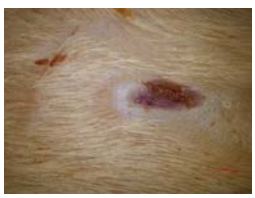
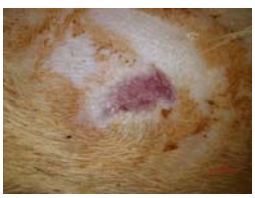
0				
1				
2				
5				
7				
10				

Figure 8. Wound images of rats infected with MRSA (Xen31) and treated with CHD-FA at 4h post infection.

Day	Rat #3L 4.6% CHD-FA	Rat #4L 0.46% CHD-FA	Rat #6R 0.046% CHD-FA	Rat#8R Untreated
0				
1				
2				
5				
7				
10				

6. Evaluation of CHD-FA to treat cutaneous wounds infected with *Pseudomonas aeruginosa* Xen5 (specific aim 2).

To assess the treatment efficacy of CHD-FA against cutaneous wounds infected with *P. aeruginosa*, 11 rats were randomized into three treatment groups (3 rats per group) and two untreated (sham) controls. Bioluminescent *P. aeruginosa* Xen5 was prepared at previously described. Wound were created and infected with 0.05 ml of *P. aeruginosa* to a final infection dose of $1x10^7$ CFU. Rats were given daily treatments of CHD-FA at 4.6, 0.46 or 0.046% in 0.025 ml volumes starting at 4 h as the MRSA study demonstrated earlier treatment time provided significantly improved wound healing.

Improved wound closure was visually observed in the highest and middle CHD-FA doses (4.6 and 0.46%) relative to the untreated control **(Fig 9)**. The greatest improvement in wound healing became evident on day 7. As expected, both the CHD-FA treated and untreated wounds were significantly healed by the experimental endpoint at day 10. Purulent discharge was observed in some of the CHD-FA treated and all of the untreated rats however, as with the MRSA treatment studies the discharge was less pronounced in the CHD-FA treated groups. Digital measurements of wound surface areas **(Table 2)** showed significant improvement in wound healing on day 7 with the highest (4.6%) and mid (0.46%) CHD-FA treatment doses. The most prominent differences were on day 7 where the average wound surface areas were 49.3%, 46.9% in contrast to the untreated control of 60.8%. The lowest CHD-FA (0.046%) treatment dose had wound surface area percentage of 61.2% on day 7, not significantly different from the untreated controls. However, less purulent discharge was observed for the low CHD-FA dose than the untreated controls. The bacterial burden assessment of the wounds at the experimental endpoint 1.0, 0.5 and 0.3 log reduction in burden for the CHD-FA treatment doses 4.6%, 0.46% and 0.046% relative to the untreated controls **(Table 3)**. As with the MRSA infection studies these promising results warrants further studies into

Conclusion

As with the MRSA infection studies there was significant reduction of the size and bacterial burden in the wound sites with high and mid CHD-FA treatment groups. We will be repeating these studies with a focus on the earlier time points to assess bacterial burden. Furthermore, we will also perform both histopathologic analyses and host gene expression profiling to better assess the cellular and molecular mechanisms during wound healing with CHD-FA.

Figure 9. Wound images of rats infected with *P. aeruginosa (Xen05)* and treated with CHD-FA at 4h post infection.

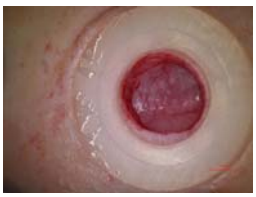
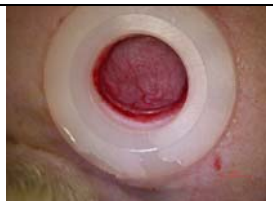
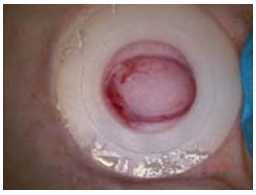
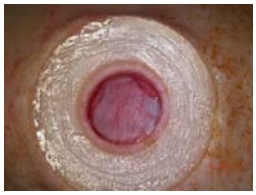
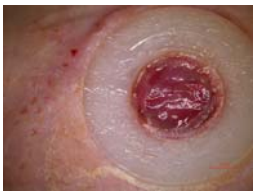
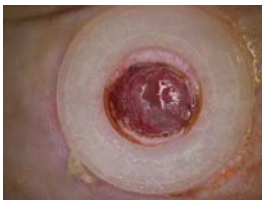
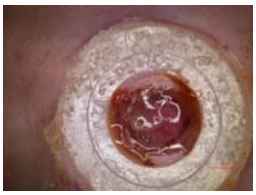
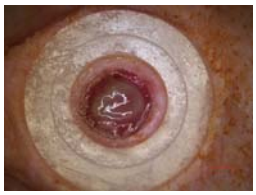
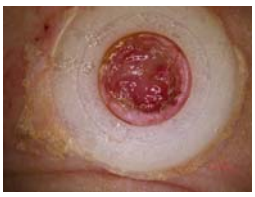
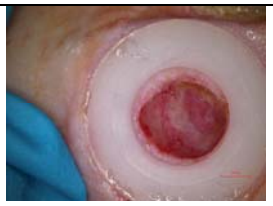
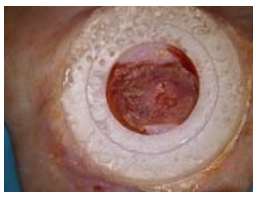
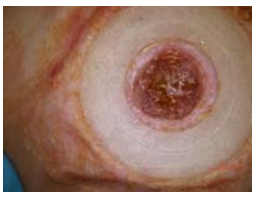
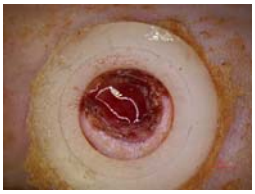
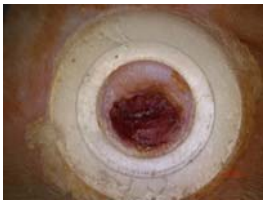
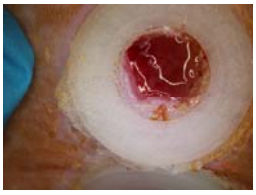
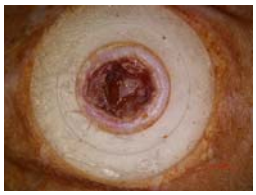
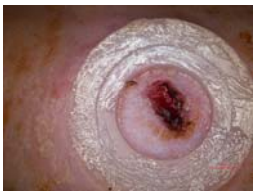
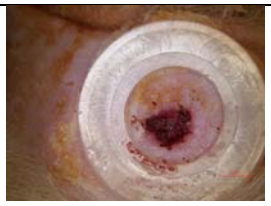
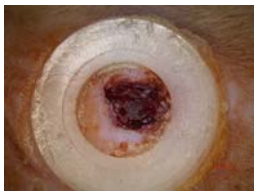
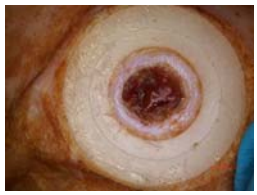
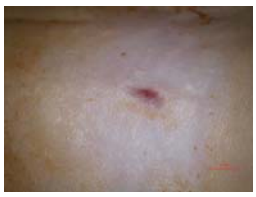
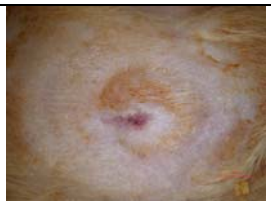
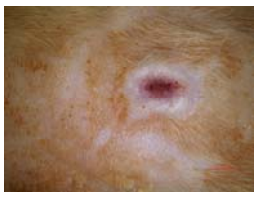
Day	Rat #2R 4.6% CHD-FA	Rat #5L 0.46% CHD-FA	Rat #8R 0.046% CHD-FA	Rat#11R Untreated
0				
1				
2				
5				
7				
10				

Table 2. Wound closure analysis of Rats infected with MRSA or *P. aeruginosa* treated with CHD-FA.

	CHD-FA Treatment Concentration (%)							
	4.6		0.46		0.046		No Tx	
Day	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD
0	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a
1	94.2%	0.08	100.0%	0.08	100.0%	0.07	100.0%	0.02
2	96.9%	0.05	98.3%	0.06	100.0%	0.05	100.0%	0.13
3	92.9%	0.11	96.9%	0.06	97.8%	0.10	100.0%	0.06
4	77.5%	0.08	83.0%	0.12	91.4%	0.06	96.2%	0.03
5	74.5%	0.08	79.5%	0.07	84.1%	0.23	93.8%	0.07
6	64.6%	0.02	66.0%	0.08	83.1%	0.11	98.7%	0.18
7	56.8%	0.08	59.1%	0.12	64.4%	0.33	70.4%	0.21
8	40.2%	0.13	46.7%	0.16	48.4%	0.27	53.1%	0.22
9	29.1%	0.13	37.0%	0.19	41.6%	0.28	44.6%	0.23
10	6.5%	0.03	25.9%	0.09	28.2%	0.20	32.2%	0.19

MRSA 24h Treatment

MRSA 4h Tx	CHD-FA Treatment Concentration (%)							
	4.6		0.46		0.046		No Tx	
Day	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD
0	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a
1	88.8%	0.08	97.3%	0.04	90.7%	0.04	93.2%	0.07
2	82.6%	0.08	97.4%	0.03	85.9%	0.03	93.5%	0.06
3	79.8%	0.08	100.0%	0.06	78.7%	0.07	89.8%	0.05
4	79.5%	0.07	98.4%	0.02	67.3%	0.09	94.5%	0.04
5	67.6%	0.14	71.5%	0.22	62.7%	0.09	86.6%	0.08
6	49.8%	0.20	51.4%	0.24	49.3%	0.14	71.7%	0.07
7	43.9%	0.20	45.3%	0.27	43.0%	0.19	64.5%	0.13
8	31.5%	0.20	39.5%	0.30	37.9%	0.16	68.2%	0.07
9	21.9%	0.17	35.5%	0.32	37.3%	0.24	49.3%	0.10
10	15.3%	0.12	10.4%	0.05	15.2%	0.10	30.2%	0.08

MRSA 4h Treatment

P. aeru 4h Tx	CHD-FA Treatment Concentration (%)							
	4.6		0.46		0.046		No Tx	
Day	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD
0	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a
1	100.0%	0.05	100.0%	0.07	100.0%	0.08	100.0%	0.05
2	96.5%	0.03	90.9%	0.07	100.0%	0.06	100.0%	0.05
3	97.4%	0.04	86.7%	0.12	104.5%	0.11	97.1%	0.05
4	95.2%	0.04	81.0%	0.11	99.6%	0.07	94.4%	0.10
5	92.6%	0.04	74.3%	0.13	92.6%	0.08	90.9%	0.11
6	66.8%	0.07	57.2%	0.14	71.5%	0.12	73.8%	0.12
7	49.3%	0.16	46.9%	0.17	61.2%	0.11	60.8%	0.16
8	45.1%	0.20	42.1%	0.14	46.4%	0.13	54.1%	0.14
9	38.2%	0.18	33.6%	0.15	36.5%	0.19	50.6%	0.18
10	11.8%	0.11	6.7%	0.04	13.5%	0.06	21.3%	0.07

P. aeruginosa 4h Treatment

Table 3. Bacterial burdens of cutaneous wounds infected with MRSA or *P. aeruginosa* at experimental endpoint (10 days, post infection)

CHD-FA conc.	Avg. Log CFU	Range	Log fold changes	Study
No Tx Control	7.0	6.6-8.2	n/a	MRSA 4H
4.60%	5.5	4.6-5.9	1.5	
0.46%	4.9	4.5-5.1	2.1	
0.046%	6.7	6.2-6.9	0.3	

CHD-FA conc.	Avg. Log CFU	Range	Log fold changes	Study
No Tx Control	5	3.8-6.2	n/a	PA 4H TX
4.60%	4	3.7-4.7	1	
0.46%	4.5	4.4-4.8	0.5	
0.046%	4.7	4.0-5.1	0.3	

Key Research Accomplishments

- **Completion of specific aim 1 of the research objectives outlined in the statement of work of the contract award.**

- **Established a reproducible cutaneous wound model in rats that can be used to assess CHD-FA to treat wound infections**

- **Performed virulence studies on bioluminescent Gram Positive and Gram Negative bacteria MRSA and *P. aeruginosa* to determine the appropriate infection dose for CHD-FA treatment studies.**

- **Obtained preliminary efficacy data on CHD-FA to treat cutaneous MRSA and *P. aeruginosa* wound infections in rats.**

- **Our initial results demonstrate CHD-FA improved wound closure (healing) and reduced microbial burdens by at least 1.5 logs relative to the untreated control groups in MRSA and 1 log with *P. aeruginosa*.**

Reportable Outcomes

We have submitted an abstract of the CHD-FA in vitro results to the 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). Dr. Perlin has also attended the Military Health System Research Symposium (MHSRS) 2013 in August to discuss the CHD-FA project. We have developed and validated a cutaneous wound model in rats for MRSA and *P. aeruginosa*. This led to collaborations with private sector companies such as Trius Pharmaceuticals and Conversion Energy Enterprises, a bio-tech firm specializing in anti-microbial medical devices. Grants have been submitted to the NIH for Partnerships for Biodefense (R01) as a sub-contract for Trius Pharmaceuticals and the NIH Centers of Excellence for Translational Research (CETR) (U19).

Conclusion

We have the completed the in vitro analysis of CHD-FA and met the objectives in specific aim 1 of the statement of work in award contract. The cutaneous wound model in rats has been established and infection models with MRSA and *P. aeruginosa* validated. Our initial results demonstrate that CHD-FA can reduce microbial burden in MRSA and *P. aeruginosa* infected wounds by one log or greater and significantly improve wound closure (healing). During the next quarter and throughout the year we will perform both histopathologic and host gene expression profiling to assess the cellular and molecular mechanisms during wound healing with CHD-FA. We will continue the wound model with other clinically important bacteria (*Acinetobacter*, *Klebsiella* and *E. coli*) and fungi (*Aspergillus*) listed in the statement of work. We will also perform the other wound infection models such as the burn model and deep tissue (thigh) model. Our studies have raised some open questions. For example, the 1-2 log reduction seen in wounds for MRSA and *Pseudomonas* raises an issue as to whether fulvic acid behaves as a bactericidal agent. The value of bioluminescent strains is still be evaluated, as the emission of light is highly sensitive to growth state of the organism and state of the wound (e.g. scab v. no-scab). Overall, we have made strong progress in the first year.

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2. Clinical and Laboratory Standards Institute. (2008) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard-Second Edition, M38-A2.* (CLSI, Wayne, PA).
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4. http://www.fulhold.com/index.php?id=96.